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STUDIES OF THE CALIFORNIA MASTITIS REACTION

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The temporary and permanent damages caused by secretory disturbances are notably high; it is estimated that in the United States of America alone the annual loss that is caused by mastitis amounts to 225 million dollars [1]. In addition to this there is the impairment of quality due to unrecognized secretory disturbances.

The importance of routine methods which are simple and quick to carry out and of as high as possible significance in determining the secretory condition of the lacteal glands is therefore quite obvious.

Whiteside [2] a number of years ago described a test for mastitis milk which is based on the reaction of such milk to caustic soda. Schalm and Norlander [3] later proposed an anionic wetting agent as specific reagent; mastitis milk when added to such wetting agents forms a gelatinous, mucilaginous reaction product whose rigidity increases in proportion to the degree of severity of the illness. Under the name of "California mastitis test" or "Schalm test" this reaction has been resorted to for numerous investigations [4-16].

The effective principle of the reaction is still unknown. The test conditions known to date vary within wide limits, but especially the choice of wetting agent.

The present investigations are primarily concerned with reviewing the optimal reaction conditions as a prerequisite for comparability of test results.

We also intended to test whether still other classes of substances besides the detergents already known are capable of giving a reaction picture analogous to that of the California test.

Substances and Methods. Reagents:

Sodium dodecyl sulfate solution. — 40 g of Na dodecyl sulfate (Serva Entwicklungslabor [Serva Development Laboratory], Heidelberg) dissolved in one liter of water and set to pH 8 with caustic soda.

Urea solution. — 48.5 g urea (Merck, purest) is dissolved in 100 ml of water (8 molar solution) and set to pH 8.

Guanidin hydrochloride solution. — 76 g guanidin hydrochloride is dissolved in 100 ml of water and brought to pH 8 (8 molar solution) with caustic soda.

Sodium salicylate solution. — Aqueous solution saturated at room temperature and set to pH 8.

Sodium desoxycholate solution. — 50 g of Na desoxycholate (Merck) is dissolved in one liter of water and set to pH 8 with caustic soda.

Heparin solution. -- Approximately 4% solution of the firm "Vitrum," Stockholm, 5000 international units per ml, set to pH 8.

Dodecyl sulfate-urea reagent. — 40 g of dodecyl sulfate is dissolved under gentle heat in the minimum amount of water necessary, set to pH 8 with caustic soda (glass electrode), and filled up to one liter with water.

Performance of the Test

In a graduated test tube or centrifuge tube equal volumes of milk and of one of the above reagents are carefully mixed by slightly tilting the tube and returning it to an upright position. Slimy, gelatinous changes, which show up especially well on the glass walls, indicate positive reaction. The more solid the gelatin appears the higher it is rated, say from + to ++++.

Experiments to Separate the Reaction Product

The reaction mixture was centrifuged about 30 minutes at 3000 rpm and the watery stratum drawn off after coagulation of the fatty top layer in the refrigerator.

In experiments with pure Na dodecyl sulfate solution this watery stratum served as starting material for nitrogen determinations by Kjeldahl's method.

The fat layer was again mixed with detergent solution and washed; separation from the wash waters was accomplished by centrifuging again. The proteins that still clung to this layer were isolated as follows: The fatty layer was mixed with about ten times its own volume of 5% Na desoxycholate solution and stirred with a glass rod until the fat part coagulated like butter. After thorough extraction with ether the watery layer

was dialyzed and lyophilized. The dry preparation is set aside for paper electrophoresis tests.

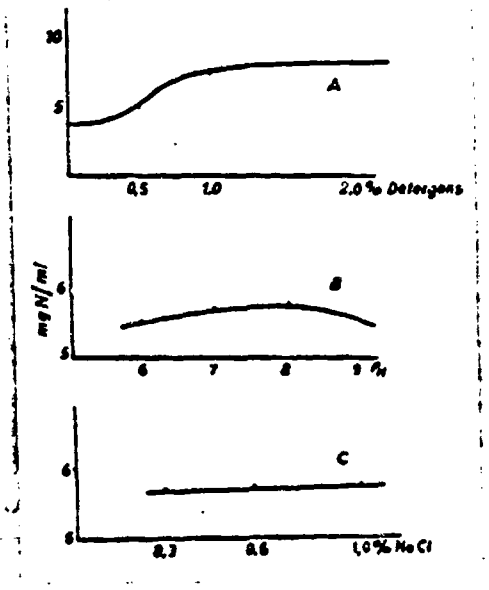
Experimental Results

A. Dependence on the Reaction Conditions

Evaluation of the California test with the testing system described is subject to considerable subjective errors in reading on the part of different observers. No exact method has yet been made known; viscosimetric measurements do not lead to reproducible results.

There are also divided opinions as to the choice of the wetting agent, its concentration, the right pH environment, and the influence of salts.

If the reaction mixture evaluated as positive is allowed to stand for a fairly long time, the fat rises to the top with the gelatin; the watery layer becomes clear and remains with no sign of precipitation. The influences that changed reaction conditions exert on the composition of this watery layer are to be seen from Figure 1; the variables are plotted against the nitrogen content.



A) Varying amount of detergent, constant pH and salt content.

B) Varying pH values, constant detergent concentration and salt content.

C) Varying salt content (NaCl), constant detergent concentration and pH.

(1 part mastitis milk - 1 part Na dodecyl sulfate solution by volume; centrifuged 30 minutes at 3000 rpm; N determination by Kjeldahl's method.)

Figure 1. Dependence of the nitrogen content of the watery stratum on the performance of the California test and separation of the fatty layer.

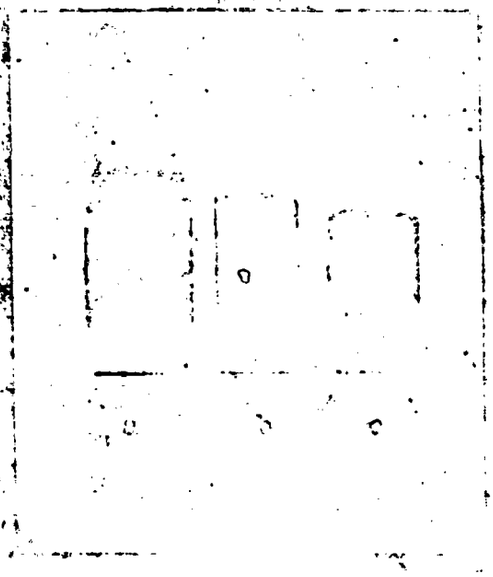
1. Dependence on the detergent concentration. — Curve A shows that with rising concentration of detergent the amount of protein in the watery stratum steadily increases until at about 2% dodecyl sulfate it becomes approximately constant. The more detergent was added, the more compact the reaction picture appeared. The experiments permit the conclusion that

increasing rigidity of the reaction picture is accompanied by a decrease in the amount of protein that goes into the cream layer. As a result the nitrogen content of the watery stratum increases.

2. Dependence on the pH value. — With constant detergent content and uniform salt concentration a slight rise in the protein content is observable between pH 6 and pH 8. The drop of the curve between pH 8 and pH 9 is steeper than the rise on the acid side. The pH optimum at 8 is clearly manifest.

3. Dependence on the salt concentration. — Curve C demonstrates that with uniform detergent concentration and maintenance of the optimum pH value the salt content has no significant influence on the protein distribution. The analysis values lie almost on a straight line, the slope of which lies within the limits of error of the method.

4. Experiments in separation of proteins from the fatty layer by paper electrophoresis. — The protein constituent in the fatty layer separated out by means of Na desoxycholate and subsequent butter-making is relatively slight; in the dried fatty layer we found approximately 90% neutral fat with an average of 3% protein. Complete separation of the lipides involves difficulties; too intensive extraction with ether lowers the protein solubility even in alkaline buffers, while too sparing treatment with ether leads to indistinct bands in electrophoresis. Figure 2 shows the electrophorograms stained with amido black,



a) of milk proteins of a mastitis milk,

b) of proteins from the fatty layer after their separation with Na desoxycholate, and

c) of the same proteins after repeated treatment with desoxycholate and ether.

Figure 2. Electrophorograms of milk proteins from mastitis milk (a) and of proteins that went over into the fatty layer in the California test (b,c).

As the figure shows, the proteins which are quite firmly bound to the fatty layer are not homogeneous; e.g. it is not possible to extract the fat completely with acetone or n-butanol.

Electrophorogram b for example clearly shows three protein components, one of which stains very strongly with amido black and the other two somewhat more weakly. Since it is possible that these protein bands represent symplexes with detergent molecules, the electrophorograms are not comparable with the picture of the milk proteins. Attempts at identifying these protein bands isolated from the fatty layer of the California test have failed up to now for methodological reasons.

5. Dependence of the California test on the pre-treatment of the milk. -- The observations of Dedié and Kielwein [4] that the skim milk of an otherwise strongly California-positive sample shows only a weak or barely recognizable reaction, while the cream centrifuged off may react very strongly, could be confirmed in our experiments.

If large quantities of positive-reacting milk are separated, e.g. in the separator attachment to the "Starmix" apparatus, the result is a strongly reacting cream and a "centrifuge" sludge sticking fast to the centrifuge dishes, which when mixed with detergent solution gives a highly viscous to clayey mass. Besides bacteria and various cell and leucocyte forms a great many fat particles are discernible in the microscopic image of this sludge. With the degree of heating the capacity of the milk to react declines; under the conditions of pasteurization the capacity to react is completely lost.

Freshly milked milk is particularly well suited to the California test; storage and aging may greatly influence the reaction picture. Storage for several days in the refrigerator conditions just as definite a decline as too warm storage; freezing the samples may lead to negative results, and freeze-drying completely destroys the capacity to react.

Treatment of a milk sample with solvents, e.g. ether, also destroys the capacity to react, and it is immaterial whether after the sample has been shaken up with ether the ether layer is drawn off after centrifuging or evaporated out in a current of air.

6. Performance of the test. — In many cases the California test has been carried out in the same way as the Whiteside test, by mixing milk and detergent in shallow dishes and then testing for slimy reaction products. With this procedure according to our experience wrong evaluations are more to be expected than when the test is carried out as suggested by Dedié and Kielwein [4] in test tubes. It is advantageous to

use graduated test tubes for this, which make the pipetting of reagents superfluous (length about 10 cm, diameter 8-9 mm).

Of the reagents listed at the beginning, the dodecyl sulfate-urea reagent proved best. The result of the sample can be read off immediately after mixing. After not more than three to four hours the cream component has collected into a stopper above a water-clear stratum. In healthy samples and those with only slight secretory disturbances the skin is only a few millimeters thick, while the stopper in strongly positive samples sometimes takes up half the column of liquid. This may be of advantage for the "readjusting" of a series of investigations.

Discussion of the Experimental Results

It has long been known that ionogenic wetting agents may react intensively with proteins [17], the proteins being either denatured, precipitated, or converted into soluble compounds. The quantitative ratio of protein to detergent and the pH environment exert a decisive influence on the result of the reaction. According to Putnam and Neurath [18] there is a close relationship between the acid-binding capacity of a protein, i.e. the number of its basic groups, and the maximum quantity of detergent bound; the presupposition for this statement, however, is the existence of strongly polar, acid groups in the detergent molecule. Acid-type compounds would accordingly be characteristic for this stage of the reaction. If the protein/detergent ratio exceeds a certain limit value, more detergent molecules are associated than can correspond to the number of basic groups; loose, van der Waals linkage forces are assumed to stabilize the compound [19]. These stages of the reaction vary depending on the kind of protein and detergent, or their constitution, so that we should have to postulate a further factor, the "specific affinity," as a measure of the "compatibility."

Almost all proteins contain a high percentage of amino acids with non-polar side chains, e.g. the isopropyl radical (valin), the butyl group (leucin), or the benzyl group (phenyl alanin). The slight affinity of these side groups for water brings about, especially when several such amino acids follow in the sequence, the pronounced inclination to recede from the water molecules into the interior of the protein molecule and to carry a part of the peptide chain with them. The strength of these "hydrophobic" compounds is said to be considerable and to make an important contribution to the stabilization of native protein configurations [20].

The addition of surface-active substances to protein solutions might obviously so obscure the contrasts between hydrophobic side chains and surrounding water molecules that the secondary and tertiary protein structures would be consequently loosened up. The superposition of all possibilities

of protein modifications leads to disruption of the structures and to denaturation, which is clearly manifested in heightened viscosity. Observations with the California reaction are in striking agreement with past experience with regard to the capacity of detergents to react with proteins.

The strong polar group in the wetting agent is a prerequisite of the California reaction; all detergents used up to now have possessed a strong anionic sulfuric acid group in direct or intermediate combination [21]. Non-ionogenic wetting agents have proved to be inactive.

The quantitative ratio of protein to detergent also influences the intensity of the Schalm test; a final concentration of the mixture of 2% detergent largely fulfills the optimum conditions. The dependence of the course of the reaction on the pH value is shown by experiments with slightly sour milk; such specimens with the addition of detergent do give a precipitation, but its slimy state comes only after neutralization with lye. For that reason in some working directions an indicator is recommended, but sometimes this addition renders evaluation of the test more difficult, so that in our experiments we have dispensed with the addition of an indicator.

The precipitation which occurs when slightly sour milk is used can also be brought into harmony with the observation that the choice of the pH environment generally decides whether protein solutions with the addition of detergents form protein precipitates or protein-detergent combinations in soluble form.

Although detergents may react with proteins in extremely varied ways and the superposition of stages of reaction occurs in complex and obscure ways, still it must be assumed that the California reaction is not evoked by a "chance phenomenon." The denaturing effect of the detergents may be compared with the effect of urea, guanidin hydrochloride, and sodium salicylate solutions. These denaturing agents, however, require a longer time for the formation of gelatinous products which are comparable with dodecyl sulfate; their reaction works better in weakly alkaline solutions, especially with substantially higher final concentrations. According to Boyd and Luck [22] the denaturing effect of urea is inhibited by dodecyl sulfate. The advantages that the combined urea-dodecyl sulfate reagent obviously offers may therefore not lie in the additive effect of the two substances. The assumption of a "modulated" course of the reaction might be supported by the observation that when pure detergent solutions are used as nearly a constant time interval as possible must be maintained between addition of the detergent and evaluation of the test. The longer reaction time is chosen the more the overall character is shifted toward strongly positive results. With the use of the combined reagent this time dependence is pushed far into the background.

The circumstance that the California reaction succeeds

only on whole milk suggests that the principal proteins of the milk do not contribute substantially to the course of the reaction. Accordingly a California-negative milk could not be changed into a positive-reacting milk by the addition of casein and its fractions nor by the addition of milk proteins.

The close link between the California effective principle and the cream stratum is obvious. The fact that heparin or desoxycholate solutions give the same result as detergent solutions indicates that lipide components must be as essential in the effective principle as protein components.

The assumption of a lipoproteide complex would agree with the experimental findings. With regard to this class it is known that water e.g. is of the greatest importance to the maintenance of the structure [23]; removal of water by freezing or freeze-drying leads to irreversible changes. These compounds are as sensitive to heat as they are to organic solvents. With detergents or denaturants they react as readily as pure proteins. In milk the lipoproteides are assumed as components of the fat-envelope substance [24]. Morton isolated lipoproteide particles from cream washed out of buttermilk [25], which contained not only about 20% phospholipides, but also nucleic acids and a hemochromogen. Because of the similarity to the microsomes from the lactating milk gland, Morton called these particles closely associated with the fat envelope "milk microsomes." It is of course still unknown within what limits the elimination of such particles may vary with the secretory state and how the tissue permeability [26] which runs parallel with the course of inflammation of the milk gland influences the quantitative relationships.

The experimental results described point to a very labile, complex system, which is capable of the California reaction only in the native state. Therein lie the special difficulties encountered in the isolation of the effective principle.

Summary

Studies were made in order to assess the significance of the results obtained in the California mastitis test. Besides the known wetting agents, urea, guanidin hydrochloride, sodium salicylate, heparin solution, and sodium desoxycholate may also react in the California test.

The use of a urea-sodium dodecyl sulfate as a reagent is recommended, since it allows better differentiation than solutions of the pure wetting agent.

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